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14. ABSTRACT

To examine the molecular mechanism by which BRCA1 participates in breast tumor suppression, we have identified that RAP80 is a BRCA1-associated protein by protein affinity purification. Here, we show the evidence that RAP80 controls BRCA1's relocation to DNA damage sites and regulates BRCA1-dependent DNA damage checkpoint function. In addition, we have identified a truncation mutation of RAP80 in an ovarian cancer cell line. Thus, our results indicate that RAP80 is a functional partner of BRCA1, and may participate in breast tumor suppression. These results has been published in Science (vol. 316, 1202-1205) and Nature Structural and Molecular Biology (Vol. 14, 716-720).

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Introduction:

Tumor suppressor gene *BRCA1* (Breast cancer susceptibility gene 1) encodes a protein of 1863 residues that contains C-terminal BRCT domain. Mutations in the BRCA1 gene (Breast cancer susceptibility gene 1) account for up to 50% of hereditary breast cancer. Clinically relevant missense mutations identified in BRCT domain abolish the tertiary structure of BRCT domain, suggesting that the BRCT domains are important for the tumor suppressor function of BRCA1[1]. Using a two-step affinity purification approach, we identified RAP80 as a new binding partner of BRCA1 BRCT domain. However, the biological function of RAP80 has not been characterized yet. We have shown that RAP80 colocalized with BRCA1 at DNA damage sites. Thus, we hypothesize that like BRCA1, RAP80 participates in DNA damage response. Also like BRCA1, RAP80 may stabilize genomic integrity and prevent tumor formation. Since BRCA1 is a breast cancer suppressor, we have planed to generate *RAP80* gene knockout mice to investigate whether RAP80 would prevent breast cancer formation in vivo. In addition, mutations in BRCA1 are responsible for familiar breast and ovarian cancers development. However, mutations of BRCA1 are rarely found in sporadic breast cancer, suggesting that may be other proteins involved in the same pathway, but not BRCA1, are mutated in these sporadic breast cancers. During the initial cloning of RAP80, we found a truncation mutant RAP80 existing in an ovarian adenocarcinoma. Interestingly, this mutant RAP80 does not localize to nuclear foci following DNA damage, suggesting that this mutant is defective in certain DNA damage responses. These observations raise the possibility that RAP80 may be more frequently mutated in sporadic breast cancer and contribute to breast caner development. Therefore, we have planed to screen for *RAP80* mutations in primary breast cancer samples.

Body:

Aim1. Investigate the role of RAP80 in DNA damage response.

RAP80 is a new binding partner of BRCA1 BRCT domain. However, the molecular mechanism of RAP80 is still unknown. Accumulated evidence suggested that BRCA1 participates in DNA damage response. Thus, we have analyzed the role of RAP80 in DNA damage induced signal transduction and cell cycle checkpoint activation.

1). Does BRCA1 BRCT domain bind phosphorylated RAP80?

Since BRCA1 BRCT domain is a phospho-protein binding domain and associates with RAP80, we have examined whether BRCA1 BRCT domain recognizes phosphor-RAP80. There are three potential binding sites of BRCA1 BRCT domain on RAP80. However, we found that BRCA1 BRCT domain does not recognize phospho-RAP80, suggesting that BRCA1 BRCT domain may not directly interact with RAP80. We continuously purified RAP80 and BRCA1 BRCT domain-associated protein by tandem affinity purification, and found that CCDC98 is the liker between BRCA1 BRCT domain and RAP80. BRCA1 BRCT domain recognizes phospho-Ser406 of CCDC98, CCDC98 associates with RAP80 through multiple regions. These finding was published in Nature Structural and Molecular Biology (Vol. 14, 716-720) by our group recently. For the detail of characterization of protein-protein interactions, please see the Figure 1 and 2 of this paper listed in Appendix.

2). Does DNA damage induce BRCA1/RAP80 complex formation?

Since BRCA1 participates in DNA damage response[2], we wonder whether DNA damage may regulate the interaction between BRCA1 and RAP80. As proposed, cells were treated with ionizing radiation to induce DNA double strand breaks, the reciprocal immunoprecipitation were performed. However, we found that the interaction between BRCA1 and RAP80 is DNA damage independent. The data has been included in a paper published in Science (vol. 316, 1202-1205). For the detail of the experiment, please see the Figure 1 of this paper as listed in Appendix.

3). Is RAP80 required for BRCA1 nuclear foci formation following DNA damage?

The BRCA1 BRCT domain targets BRCA1 to DNA damage site[3]. Since RAP80 is a BRCA1 BRCT domain interacting protein, we reasoned that RAP80 might regulate BRCA1's translocation to DNA damage site. As shown in the Figure 2 of the paper published in Science (vol. 316, 1202-1205) and the Figure 4 of the paper published in Nature Structural and Molecular Biology (Vol. 14, 716-720), RAP80 is required for BRCA1 nuclear foci formation following DNA damage. These results demonstrated that RAP80 is a functional partner of BRCA1 in the DNA damage response.

4). Does BRCA1/RAP80 complex regulate downstream Chk1 kinase activity following DNA damage?

Chk1 is a downstream kinase of BRCA1 in response to DNA damage[4]. And Chk1 activation is important for intra-S phase and G2/M phase checkpoint activation. Since Ser345 phosphorylation is critical for Chk1 activation, we used this phosphorylation event as a surrogate marker to examine Chk1 activation. As shown in the Figure 4 of the paper published in Science (vol. 316, 1202-1205), we demonstrated that RAP80 regulates downstream Chk1 kinase activation following DNA damage. These results suggest that RAP80 plays an important role in the DNA damage-induced signal transduction and checkpoint activation.

In summary, we have finished all the proposed experiments listed in Aim1. These experimental results have been included in two papers published in Science and Nature Structural and Molecular Biology.

Aim2. Study whether RAP80 is involved in breast cancer genesis in vivo.

To explore whether *RAP80* is a breast cancer suppressor gene, we have planed to generate *RAP80* knockout mice, and examined whether loss of RAP80 will increase breast cancer incidence in mice. An ES cell line, RRN158, was purchased from Bay Genomics. In RRN158 ES cell line, *RAP80* gene was disrupted by a neo gene selection cassette inserted between exon 1 and exon 2 of *RAP80* (Figure 1A). And we have mapped the exact insertion site by genomic PCR and DNA sequencing (data not shown). The ES cells were injected into C57BL/6 blastocysts to generate chimeric mice. The chimeric mice were then crossed back with C57BL/6 mice to obtain RAP80 +/– mice. RAP80 +/– mice were intercrossed,

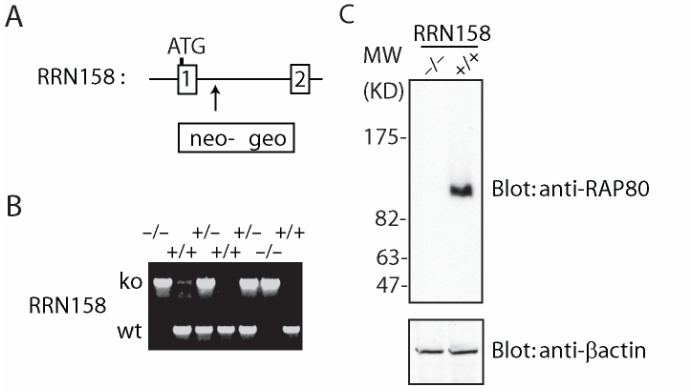
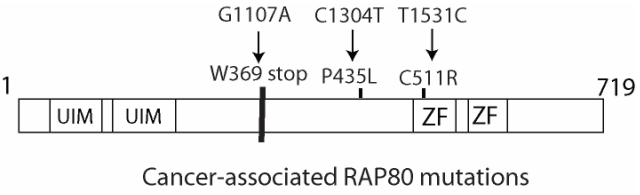


Figure 1. Generating RAP80 deficiency mice. (**A**) A neo cassettes was inserted into RAP80 gene to disrupt RAP80 expression in RRN158 ES cell line. (**B**) 4 sets of PCR primers were designed to distinguish between wild-type allel (wt) and knock-out allel (ko) of RAP80 gene. The gemonic DNA from mouse tails was used as the template to perform PCR genotyping. –/– mice were obtained in F2 generation. (**C**) RAP80 is not expressed in RAP80 –/– MEFs. RAP80 +/+ and –/–MEFs were collected and lyzed. Cell lysates were blotted with anti-mouse RAP80 Ab.

and RAP80 –/– mice were obtained. We confirmed that RAP80 was not expressed in RAP80 –/– mice (Figure 1B and C). RAP80 –/– mice are viable, and we have not found any obvious developmental defects yet. Although the viability phenotype of RAP80 -/- mice is different from BRCA1 null mice that die in early embryogenesis[5], it resembles that of BRCA1 BRCT domain deletion mice which are viable. Since BRCA1 BRCT domain deletion mice develop spontaneous tumors, especially breast tumors in their late life stage[6], we plan to monitor tumor incidence in RAP80 deficient mice.

Aim3. Examine whether RAP80 is downregulated or mutated in human breast cancer samples.

A) Screen RAP80 mutations in sporadic breast cancer.



During initial cloning RAP80 gene, we found a missense mutation on RAP80 cDNA from an ovarian adenocacinoma cDNA library. This mutated cDNA encodes an N-terminus truncation product, which disrupts the interaction with BRCA1 and fails to localize to nuclear foci following DNA damage. Therefore, it is possible that RAP80 may be mutated or dysregulated in human cancers and thus contributes to the development of sporadic breast cancer. We have designed 15 pairs of primers and screened all 14 exons of RAP80 gene in 50 breast cancer cell lines and 20 ovarian cancer cell lines. We have identified two homozygous missense mutations and one truncation mutation. The two homozygous missense mutations are C1304T (Pro435-to-Leu) in SUM229 cells, and T1531C (Cys511-to-Arg) in SUM149 cells. The truncation mutation is G1107A in TOV21G cells, which generates a stop codon at Trp369. Thus, our results indicate that RAP80 may be mutated in a portion of sporadic breast cancer patients. These results may be important for the breast cancer prevention and early diagnosis.

B) Examine RAP80 expression in sporadic breast cancer.

We have not yet observed any significant downregulation of RAP80 in sporadic breast cancers.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have demonstrated that RAP80 is a functional partner of BRCA1 in the DNA damage response. RAP80 targets BRCA1 to DNA damage sites.

2. We have found that RAP80 regulates DNA damage-induced signal transduction and cell cycle checkpoint activation.

3. We have identified that CCDC98 is the linker between BRCA1 and RAP80.

4. We have generated RAP80 knock-out mice.

5. We have screened RAP80 gene mutations in sporadic breast and ovarian cancers, and identified several RAP80 gene mutations.

REPORTABLE OUTCOMES:

Two manuscripts have been published as listed below.

1. "Ubiquitin-Binding Protein RAP80 Mediates BRCA1-Dependent DNA Damage Response"
Kim, H., Chen, J. * and Yu, X*. (2007). Ubiquitin-binding protein RAP80 mediates BRCA-dependent DNA damage responses. *Science*. 316, 1202-1205.
(* co-corresponding authors)

2. "CCDC98 targets BRCA1 to DNA damage sites"
Liu, Z., Wu, J., and Yu, X. (2007). CCDC98 targets BRCA1 to DNA damage sites. *Nature Structural and Molecular Biology*. 14, 716-720.

CONCLUSION:

Taken together, we have successfully finished the proposed experiments in Aim1, which results two published papers. Currently, we are focusing on Aim2 and 3. We have done or are performing the proposed assays listed in the original Statement Of Work, and has met the timeline set in the original Statement Of Work.

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APPENDICES: Please see attached papers.

SUPPORTING DATA: Supporting data were incorporated in the Body.

class of DNA repair proteins that uses tandem UIM domains as part of its recruitment to DSBs. In contrast to IRIF formation, incomplete BRCA1 localization at laser-induced DSBs still occurs in the absence of γH2AX (*22*), MDC1 (*17*), or RAP80 (Fig. 3, E and F). These findings may reflect the fact that BRCA1/BARD1 heterodimers are components of multiple distinct complexes (*9*) and that each may access DSBs by different mechanisms. Taken together, these findings strongly suggest an essential role for ubiquitin recognition by a specific BRCA1 complex in the response to DSB formation. In addition, the synthesis and turnover of certain polyubiquitinated structures by BRCA1 E3 and BRCC36 DUB activities, respectively, may contribute to BRCA1-dependent DSB repair.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5828/1198/DC1
Materials and Methods
Figs. S1 to S4
References and Notes

4 January 2007; accepted 10 April 2007
10.1126/science.1139516

Ubiquitin-Binding Protein RAP80 Mediates BRCA1-Dependent DNA Damage Response

Hongtae Kim,[1] Junjie Chen,[1]* Xiaochun Yu[2]*

Mutations in the breast cancer susceptibility gene 1 (*BRCA1*) are associated with an increased risk of breast and ovarian cancers. BRCA1 participates in the cellular DNA damage response. We report the identification of receptor-associated protein 80 (RAP80) as a BRCA1-interacting protein in humans. RAP80 contains a tandem ubiquitin-interacting motif domain, which is required for its binding with ubiquitin in vitro and its damage-induced foci formation in vivo. Moreover, RAP80 specifically recruits BRCA1 to DNA damage sites and functions with BRCA1 in G_2/M checkpoint control. Together, these results suggest the existence of a ubiquitination-dependent signaling pathway involved in the DNA damage response.

Despite developing various DNA lesions generated during DNA replication or after exposure to environmental agents, cells normally maintain their genomic integrity and prevent neoplastic transformation because of the existence of several cell cycle checkpoints and DNA repair systems (*1*–*3*). Many proteins [including the protein kinase ataxia-telangiectasia mutated (ATM), γ-H2AX, mediator of DNA damage checkpoint protein 1 (MDC1), Nijmegen breakage syndrome 1 (NBS1), BRCA1, and checkpoint kinases 1 and 2 (Chk1 and Chk2)] are involved in the ionizing radiation (IR)–induced DNA damage response pathway (*4*). ATM is recruited to and activated at the sites of DNA breaks. Activated ATM transduces DNA damage signals to downstream proteins, including BRCA1. *BRCA1* encodes a tumor suppressor gene that is mutated in ~50% of hereditary breast and ovarian cancer patients (*5*, *6*). The human BRCA1 protein contains an N-terminal RING finger domain that has intrinsic E3 ubiquitin ligase activity and tandem BRCA1 C-terminal (BRCT) domains at its C terminus, which are phosphoserine- or phosphothreonine-binding motifs (*7*–*9*). Many disease-causing mutations are detected within these two regions of BRCA1.

Although BRCA1 is recruited to the sites of DNA breaks and participates in cell cycle checkpoint control, it remains obscure how the recruitment of BRCA1 is controlled in the cell. We purified BRCA1-BRCT domains from human leukemia K562 cells stably expressing this protein with N-terminal S-tag, Flag epitope, and streptavidin-binding peptide (SFB) triple tags (SFB-BRCA1-BRCT). We detected three specific bands that eluted with the SFB-BRCA1-BRCT domain but not with the SFB-BARD1-BRCT domain (Fig. 1A), where BARD1 signifies the BRCA1-associated RING domain protein 1. Mass spectrometry analysis revealed that these three proteins (respectively) are BRCA1-associated C-terminal helicase (BACH1), C-terminal binding protein–interacting protein (CtIP), and RAP80. BACH1 and CtIP are two known BRCA1 BRCT domain-binding proteins (*9*, *10*). RAP80 was originally identified as a retinoid-related testis-associated protein (*11*). The physiological function of RAP80 is unknown. We first confirmed the association between RAP80 and BRCA1 both in vitro and in vivo (Fig. 1B and fig. S1) (*12*). The interaction between BRCA1 and RAP80 remained the same before or after DNA damage (Fig. 1C).

BRCA1 relocalizes to sites of DNA breaks in cells exposed to IR. Immunostaining showed RAP80 to be evenly distributed in the nucleoplasm in normal cells (Fig. 2A). However, after exposure of cells to IR, RAP80 relocalized to foci that colocalized with γ-H2AX and BRCA1 (Fig. 2, A and B). RAP80 also associated with chromatin only in cells exposed to IR (Fig. 2C). Together, these data indicate that the localization of RAP80, like that of BRCA1, is regulated in response to DNA damage.

RAP80 isolated from irradiated cells migrated more slowly during SDS–polyacrylamide gel electrophoresis (SDS-PAGE) than did RAP80 isolated from unirradiated cells. Moreover, phosphatase treatment reversed the slow mobility of RAP80 prepared from irradiated cells (Fig. 2D), indicating that RAP80 may be phosphorylated in cells exposed to IR. We confirmed this using a phosphospecific antibody raised against a phosphorylation site that we identified (Ser^{101}; fig. S2). The ATM protein kinase is activated in response to DNA damage and phosphorylates many proteins involved in the DNA damage response. Treatment of cells with two different ATM kinase inhibitors, wortmannin and caffeine, abolished the IR-induced mobility shift of RAP80 (fig. S3A). The mobility shift of RAP80 was only observed in cells expressing wild-type (WT) ATM but not in ATM-deficient cells (Fig. 2E). These data suggest that ATM is required for damage-induced phosphorylation of RAP80.

The accumulation of RAP80 to the sites of DNA breaks depended on MDC1 and H2AX (Fig. 2, F and G) but not on NBS1, p53 binding

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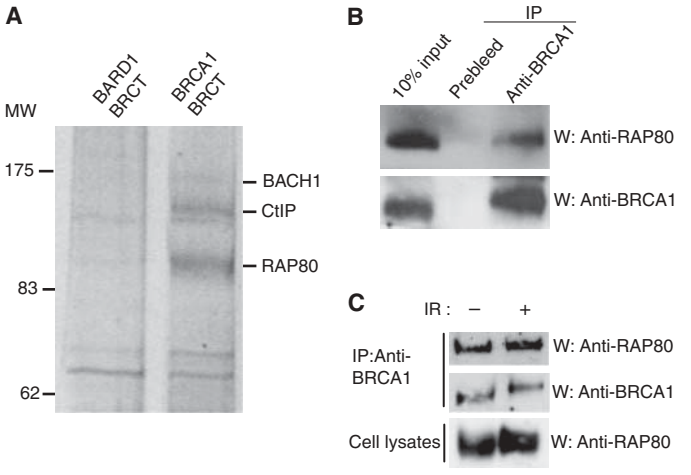
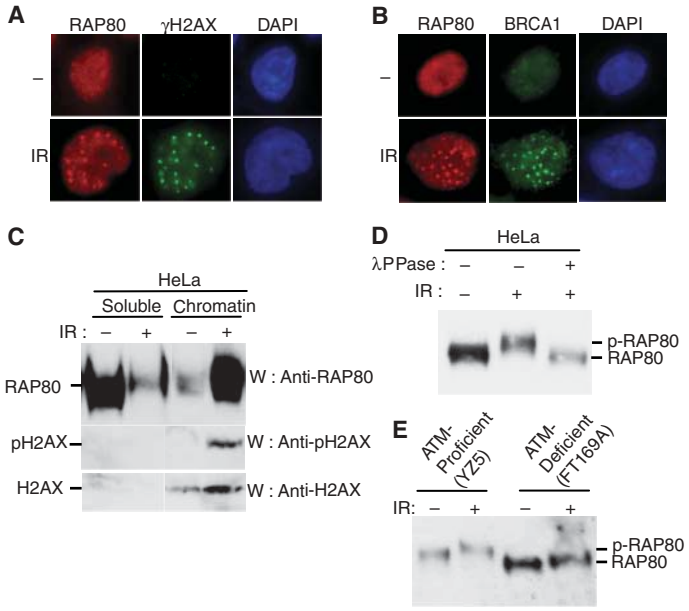


Fig. 1. Identification of RAP80 as a BRCA1-binding protein. (**A**) Silver staining of affinity-purified BRCA1-BRCT complexes. The cell extracts prepared from K562 cells stably expressing SFB-BRCA1-BRCT or SFB-BARD1-BRCT were subjected to two rounds of affinity purification. Final elutes were analyzed by SDS-PAGE and visualized by silver staining. The specific bands were excised from the silver-stained gel, and the peptides were identified by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry. Lines indicate protein bands corresponding to BACH1, CtIP, and RAP80. (**B**) The interaction between endogenous BRCA1 and RAP80. We performed immunoprecipitation (IP) reactions using preimmune serum or antibody to BRCA1. The immunoprecipitates were subjected to immunoblotting analyses with antibodies to BRCA1 or RAP80. (**C**) The interaction between BRCA1 and RAP80 before and after exposure of cells to IR. Lysates prepared from mock-treated or irradiated 293T cells were immunoprecipitated with antibody to BRCA1. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with the indicated antibodies (top two lanes). The amount of endogenous RAP80 in cells before and after radiation was shown in the bottom lane.

protein 1 (53BP1), or BRCA1 (fig. S3, B to D). When we reduced endogenous RAP80 expression using RAP80 small interfering RNAs (siRNAs), we still detected damage-induced foci formation of MDC1, γ-H2AX, and 53BP1. However, no BRCA1 foci were present in these RAP80-depleted cells (Fig. 2H), suggesting that RAP80 acts upstream of BRCA1 and is required for the accumulation of BRCA1 to sites of DNA breaks.

We also determined which regions of RAP80 are important for its focus localization. Full-length and several internal deletion mutants of RAP80 localized to nuclear foci in cells with DNA damage, whereas RAP80D1 and RAP80D2 did not (Fig. 3A and fig. S4A). Because RAP80D1 and RAP80D2 are the only two internal deletion mutants that lack the two putative ubiquitin-interacting motifs (UIMs) (*13*), these results imply that the region containing UIMs may be required for RAP80 localization to DNA damage foci. The putative UIMs in RAP80 largely match with the UIM consensus sequence (fig. S4B). To determine whether the tandem RAP80 UIMs indeed bind to ubiquitin, we used a ubiquitin–



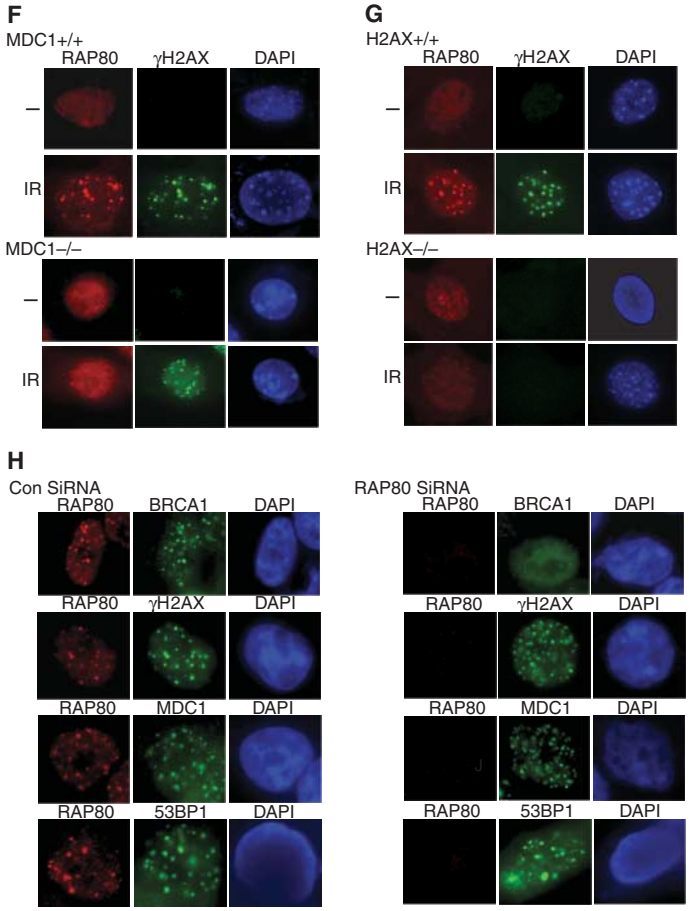


Fig. 2. Localization and phosphorylation of RAP80 in cells exposed to IR. DNA damage–induced RAP80 foci formation and its colocalization with γ-H2AX (**A**) and BRCA1 (**B**) are shown. DAPI, 4′,6′-diamidino-2-phenylindole. Mock-treated or irradiated 293T cells were fixed and stained with monoclonal antibodies to γ-H2AX or BRCA1 and polyclonal antibody to RAP80. (**C**) Association of RAP80 with chromatin after DNA damage. The soluble and chromatin fractions were prepared from mock-treated or irradiated 293T cells and subjected to Western blot analysis with antibodies to RAP80 (top lanes), phosphorylated H2AX (p-H2AX) (middle lanes), and H2AX (bottom lanes). (**D**) Phosphorylation of RAP80 after DNA damage. Lysates prepared from control or irradiated HeLa cells were immunoprecipitated with antibody to RAP80 and then incubated with or without λ phosphatase for 1 hour at 30°C. λPPase, λ protein phosphatase. The samples were subjected to immunoblotting with antibody to RAP80. (**E**) Requirement of ATM for IR-induced phosphorylation of RAP80. ATM-deficient FT169A cells and cells reconstituted with WT ATM (YZ5) were exposed to IR. Immunoprecipitation and immunoblotting were performed as described in (D). (**F** and **G**) Dependence of DNA damage–induced RAP80 foci formation. MDC1$^{+/+}$ and MDC1$^{-/-}$ mouse embryo fibroblasts (MEFs) (F) and H2AX$^{+/+}$ and H2AX$^{-/-}$ MEFs (G) were exposed to IR. The immunostaining experiments were performed as described in (A). (**H**) Requirement of RAP80 for damage-induced BRCA1 foci formation. Control (con) or RAP80 siRNA-transfected 293T cells were exposed to IR. Immunostaining was conducted with monoclonal antibodies to BRCA1, MDC1, 53BP1, or γ-H2AX and polyclonal antibody to RAP80.

glutathione *S*-transferase fusion protein (Ubi-GST). Ubi-GST specifically bound to the WT RAP80 but not to RAP80 lacking the two putative UIMs (RAP80D1; Fig. 3B). We also tested the binding of WT or mutant RAP80 UIMs [mutation of Ala^{88}→Gly^{88} (A88G) (*14*) and S92A in the first UIM and A113G and S117A in the second UIM] with Ubi-GST. The Ubi-GST specifically interacted with RAP80 UIM but not with the UIMs containing point mutations (Fig. 3B). We further checked whether point mutants within RAP80 UIMs would disrupt RAP80 foci formation in vivo. WT RAP80 and the RAP80P4 mutant (mutation of the linker region between two UIMs) formed detectable damage-induced nuclear foci, whereas the RAP80P1, RAP80P2, and RAP80P3 point mutants did not (Fig. 3C and fig. S4A). RAP80P1, RAP80P2, and RAP80P3 contain mutations within the first UIM (A88G and S92A), the second UIM (A113G and S117A), or both UIMs (A88G, S92A, A113G, and S117A), respectively. Therefore, the ubiquitin-binding activity of RAP80 correlates with its ability to localize to damage-induced foci in vivo. Like RAP80, the *Homo sapiens* DnaJ1A (HSJ1A) protein localizes to nuclei and also contains two UIMs. However, full-length HSJ1A or a construct containing the HSJ1A UIM region did not form nuclear foci in cells with DNA damage (fig. S4C). Thus, the ability to form nuclear foci is specific for the RAP80 UIM region. Notably, RAP80 UIMs bind specifically to Lys^{63}-linked but not to Lys^{48}-linked polyubiquitin chains in vitro (fig. S5).

Cells carrying BRCA1 mutants display increased sensitivity to IR and defective G_2/M checkpoint control (*15*). We examined whether the loss of the RAP80 would result in similar defects in the DNA damage response. Both RAP80 siRNAs that we synthesized efficiently decreased RAP80 expression in cells (Fig. 4A). Using a previously established G_2/M checkpoint assay (*16*), we showed defective G_2/M checkpoint control in RAP80-depleted cells (Fig. 4B). Similar G_2/M checkpoint defects were also observed in BRCA1- or CtIP-depleted cells (fig. S6). The protein kinase Chk1 is required for the G_2/M checkpoint control (*17*, *18*) and acts downstream of BRCA1 in response to IR (*19*, *20*). If RAP80 functions upstream of BRCA1, we would expect a defective Chk1 activation in RAP80-depleted cells. This is indeed the case (Fig. 4C). RAP80-depleted cells were also more sensitive to radiation than control cells (Fig. 4D). These data together indicate that RAP80 acts upstream of BRCA1 and specifically regulates BRCA1 functions after DNA damage.

Exactly how RAP80 is recruited to DNA damage sites is still unknown. Because RAP80 UIMs bind directly to ubiquitin in vitro, we reason that one or several ubiquitinated proteins might bind RAP80 and recruit RAP80 to the DNA damage sites. There are several proteins known to be ubiquitinated and localized to the sites of DNA damage (*21*–*23*). One of them is Fanconi anemia complementation group D2 (FANCD2). However, RAP80 foci still form normally after irradiation in FANCD2-deficient cells (fig. S7), suggesting that there may be other as-yet-unidentified ubiquitinated proteins that act

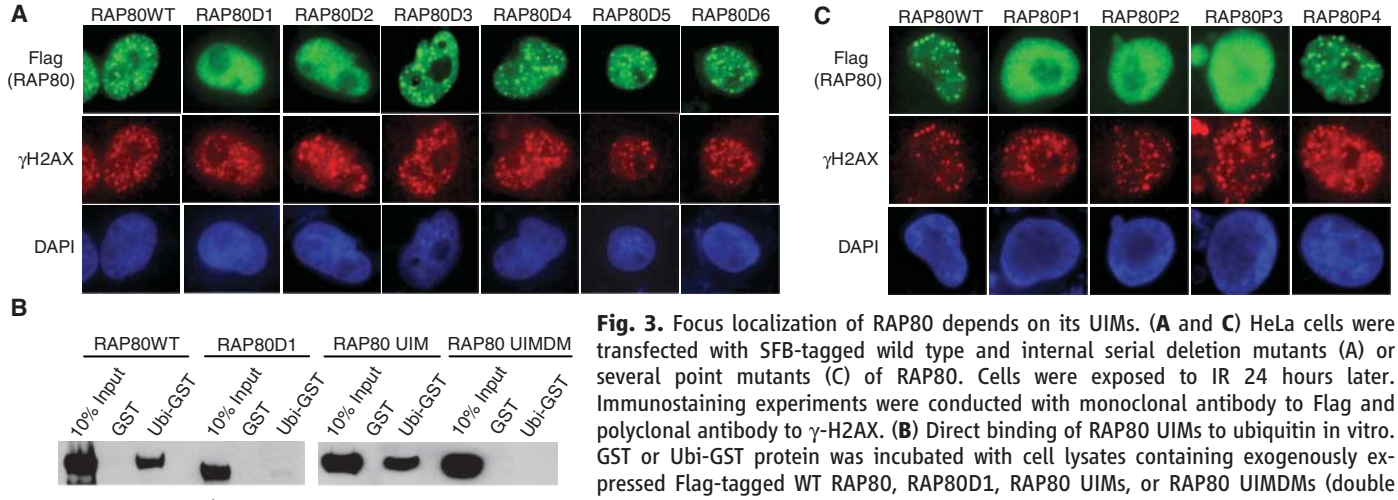


Fig. 3. Focus localization of RAP80 depends on its UIMs. (**A** and **C**) HeLa cells were transfected with SFB-tagged wild type and internal serial deletion mutants (A) or several point mutants (C) of RAP80. Cells were exposed to IR 24 hours later. Immunostaining experiments were conducted with monoclonal antibody to Flag and polyclonal antibody to γ-H2AX. (**B**) Direct binding of RAP80 UIMs to ubiquitin in vitro. GST or Ubi-GST protein was incubated with cell lysates containing exogenously expressed Flag-tagged WT RAP80, RAP80D1, RAP80 UIMs, or RAP80 UIMDMs (double mutations in the UIMs). After extensive washing, the bound RAP80 proteins were analyzed by immunoblotting with antibody to Flag.

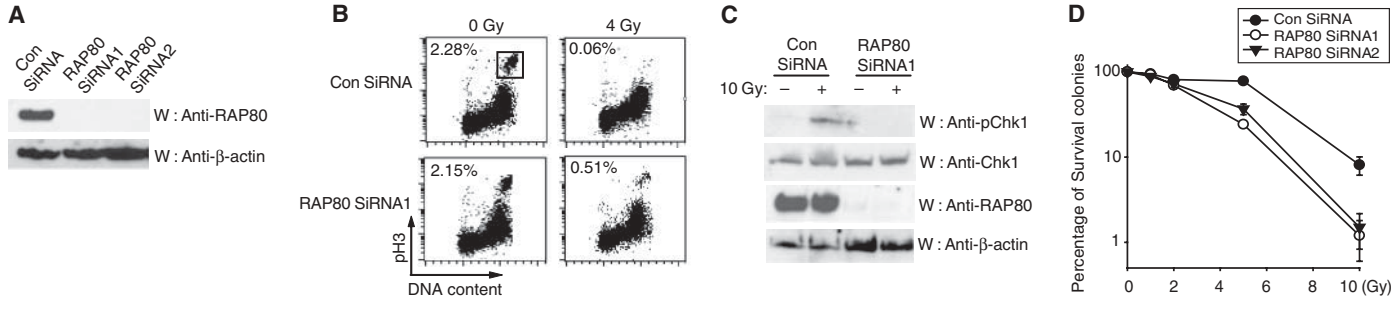


Fig. 4. Requirement of RAP80 for the IR-induced G_2/M checkpoint. (**A**) Western blot analysis for RAP80 expression level. RAP80 protein levels were analyzed by immunoblotting with antibodies to RAP80 with the use of control or RAP80 siRNA-transfected cell lysates. (**B**) G_2/M checkpoint in RAP80-depleted cells. HeLa cells transfected with control or RAP80 siRNAs were exposed to 0 or 4 grays (Gy) of IR. Cells were incubated for 1 hour before fixation and subjected to staining with antibody to phosphorylated histone H3 (pH3) and propidium iodide. The percentages of mitotic cells were determined by fluorescence-activated cell sorting analysis. The boxed area in the top left panel indicates mitotic cells. (**C**) Requirement of RAP80 for Chk1 phosphorylation after DNA damage. Control or RAP80 siRNA-transfected HeLa cells were exposed to IR. Cells were harvested 2 hours later, and lysates were immunoblotted with indicated antibodies. (**D**) Radiation sensitivity of cells lacking RAP80. HeLa cells were transfected with control or RAP80 siRNAs. Cells were counted and irradiated with various doses of IR. Percentages of surviving colonies were determined 11 days later. These experiments were performed in triplicate, and the results represent the average of two or three independent experiments. Error bars indicate SD for different doses of irradiation.

early in DNA damage response and regulate RAP80 localization.

Many cell cycle checkpoint proteins, including ATM, Chk2, BRCA1, and p53, play critical roles in the maintenance of genomic stability. Their mutation often results in increased tumor incidence, highlighting the importance of the integrity of DNA damage pathways in tumor suppression. As a BRCA1-associated protein involved in DNA damage checkpoint control, RAP80 may also function as a tumor suppressor and be dysregulated or mutated in human patients. Future genetic studies will allow us to test this possibility.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5828/1202/DC1
Materials and Methods
Figs. S1 to S9
References

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How Synaptotagmin Promotes Membrane Fusion

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Synaptic vesicles loaded with neurotransmitters are exocytosed in a soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)–dependent manner after presynaptic depolarization induces calcium ion (Ca^{2+}) influx. The Ca^{2+} sensor required for fast fusion is synaptotagmin-1. The activation energy of bilayer-bilayer fusion is very high (≈40 k_BT). We found that, in response to Ca^{2+} binding, synaptotagmin-1 could promote SNARE-mediated fusion by lowering this activation barrier by inducing high positive curvature in target membranes on C2-domain membrane insertion. Thus, synaptotagmin-1 triggers the fusion of docked vesicles by local Ca^{2+}-dependent buckling of the plasma membrane together with the zippering of SNAREs. This mechanism may be widely used in membrane fusion.

At the synapse, neurotransmitter release is mediated by the Ca^{2+}-induced fusion of transmitter-loaded synaptic vesicles with the presynaptic plasma membrane. The plasma membrane–localized target (t)-SNAREs ([soluble *N*-ethylmaleimide–sensitive factor attachment protein 25 (SNAP-25) and syntaxin-1)] and the vesicle (v)–localized v-SNARE (synaptobrevin) and synaptotagmin-1 (syt1) are involved in the Ca^{2+}-triggered fusion of synaptic vesicles with the plasma membrane (*1*). The three SNAREs are believed to bring the two membranes destined for fusion into close apposition. Syt1 has been shown to be the Ca^{2+} sensor responsible for Ca^{2+}-triggered fusion (*2*), but the molecular mechanism by which syt1 accomplishes this is not fully understood. Syt1 is a vesicle-localized transmembrane protein with two cytoplasmic C2 domains, C2A and C2B (Fig. 1A). The C2A and the C2B domains each bind Ca^{2+}, which enables them to interact with membranes (*3*, *4*). This activity is implicated in the triggering of membrane fusion (*5*, *6*). In addition, Ca^{2+}-dependent and -independent interactions between syt1 with SNAREs have been shown (*7*).

The fusion of two membranes is now widely believed to occur through a hemifusion intermediate (*8*). For hemifusion to occur, high energy barriers must be overcome, which are thought to be related to the curvature deformations generated within the membranes during stalk formation and subsequent stages of membrane merging (*8*, *9*). Syt1 has been shown to trigger Ca^{2+}-induced fusion and bind to membranes in a Ca^{2+}-dependent manner, and thus we investigated whether it could promote membrane fusion and, consequently, exocytosis, by affecting local membrane curvature.

Ca^{2+} binding by syt1 is mediated by a series of conserved aspartate residues that line pockets on one end of each of the C2A and C2B domains (*3*, *10*) (Fig. 1A). We used a syt1 construct lacking the transmembrane domain but having the double C2 domain module (C2AB) (*11*). Ca^{2+} binding allows the C2A and C2B domains to interact with negatively charged phospholipids such as phosphatidylserine (PtdSer) and phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] (*12*, *13*) (fig. S1). This interaction results in the insertion of four loops (two from each of the C2 domains) into the lipid bilayer (*14*, *15*). M173, F234, V304, and I367 (*16*) located on the tips of the membrane-binding loops (Fig. 1A) penetrate to a third of the lipid monolayer depth (*15*). This kind of hydrophobic-loop insertion should generate a tendency for the monolayer to bend to relieve the tension created by the insertion. If syt1 contributes to spontaneous membrane curvature (*8*), the closer the membrane curvature is to that preferentially produced by syt1, the stronger the syt1 affinity for membrane binding should be. Conversely, addition of syt1 to initially flat membranes should induce a positive curvature.

We incubated liposomes of different sizes, and, consequently, of different curvatures, with syt1 C2AB domains in the presence and absence of 1 mM Ca^{2+}. The binding of syt1 to membranes was monitored by a cosedimentation assay (Fig. 1B). Syt1 showed a clear preference for binding smaller liposomes (Fig. 1, Bii and C). This effect was observed only in the presence of Ca^{2+}, whereas the Ca^{2+}-independent interaction of syt1 with liposomes was size independent (Fig. 1Bi). This positive-curvature preference was largely lost when we increased the strength of interaction of syt1 with the membrane by elevating the PtdSer content in the liposomes from 15 to 25% (Fig. 1D). Likewise, the binding to Folch liposomes, which are rich in PtdSer, was largely curvature independent. The syt1 C2AB domain

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nature structural & molecular biology

CCDC98 targets BRCA1 to DNA damage sites

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Breast cancer-1 (BRCA1) participates in the DNA damage response. However, the mechanism by which BRCA1 is recruited to DNA damage sites remains elusive. Recently, we have demonstrated that a ubiquitin-binding protein, RAP80, is required for DNA damage–induced BRCA1 translocation. Here we identify another component, CCDC98, in the BRCA1–RAP80 complex. CCDC98 mediates BRCA1's association with RAP80. Moreover, CCDC98 controls both DNA damage–induced formation of BRCA1 foci and BRCA1-dependent G2/M checkpoint activation. Together, our results demonstrate that CCDC98 is a BRCA1 binding partner that mediates BRCA1 function in response to DNA damage.

BRCA1 is a nuclear polypeptide of 1,863 amino acid residues. It contains a ring domain at the N terminus and a BRCT domain at the C terminus. The ring domain associates with the BARD1 ring domain, forming a heterodimer that functions as an E3 ubiquitin ligase[1–6]. We and others have demonstrated that the BRCT domain is a phosphoprotein-binding domain[7–13].

Mutations of the *BRCA1* gene account for more than 50% of familial breast cancers and 20%–30% of inherited ovarian cancers. The accumulated evidence suggests that BRCA1 participates in the DNA damage response and protects genomic integrity after both endogenously and exogenously induced DNA damage, especially double-strand breaks[14–19].

In response to DNA double-strand breaks, a group of evolutionarily conserved phosphatidylinositol-3 kinases, including ATM, ATR and DNAPK, are activated. The activated kinases initiate signal cascades to stop cell-cycle progression, allowing DNA lesions to be repaired[20,21]. BRCA1 has been shown to participate in this process, known as the DNA damage checkpoint. Upon DNA damage, BRCA1 is phosphorylated by ATM and ATR[22–25], and it regulates the downstream CHK1 kinase, whose activity controls the progression of S phase and the G2/M transition during the cell cycle[26,27]. The role of BRCA1 in the DNA damage response is further supported by its subnuclear localization. In the presence of DNA double-strand breaks, BRCA1 forms nuclear foci that colocalize with phosphorylated histone H2AX (γH2AX)[28–30], a marker of DNA damage sites[31]. Notably, a truncated form of BRCA1 without the C-terminal BRCT domain does not form DNA damage–induced foci, suggesting that the BRCT domain is required for BRCA1 subnuclear localization[30].

Previously, we and others have characterized two BRCA1-BRCT domain–binding proteins, BACH1 and CtIP[7,32–39]. However, neither of them is required for formation of BRCA1 foci in response to DNA double-strand breaks. Here, by using protein affinity purification, we identify another BRCA1-BRCT domain–binding protein, CCDC98. CCDC98 is a 409-residue polypeptide with an N-terminal coiled-coil domain and a nuclear localization sequence. This protein not only targets BRCA1 to DNA damage sites but also bridges BRCA1 and its functional partner RAP80, which we and others have recently characterized[40–42]. Moreover, CCDC98 participates in the BRCA1-dependent G2/M checkpoint after DNA damage. Thus, our results further delineate the molecular mechanism by which BRCA1 functions in DNA damage checkpoint control.

RESULTS

CCDC98 binds the BRCA1-BRCT domain

Recently, we and others have reported that RAP80 functions as a partner of BRCA1-BRCT in response to DNA damage[40–42]. Although endogenous RAP80 associates with BRCA1 to form a complex, we could not detect direct binding between these two proteins *in vitro* (data not shown). Thus, we hypothesized that mediators link BRCA1 and RAP80. To identify potential mediators, we added S–Flag–streptavidin-binding peptide (SBP) triple tags to both the BRCA1-BRCT domain and RAP80. Using sequential chromatography, we identified nine CCDC98 peptides, covering 21% of CCDC98's sequence, among purified BRCT domain–binding proteins. In a similar screen for RAP80-associated proteins, we found 18 CCDC98 peptides that covered 30% of its sequence.

To confirm the association among CCDC98, BRCA1 and RAP80, we performed coimmunoprecipitation assays. As expected, endogenous CCDC98 coimmunoprecipitated with BRCA1 and with RAP80 (**Fig. 1**), suggesting that the three form a complex. As the BRCA1–RAP80 complex is important in the DNA damage response, we examined whether DNA damage induces association of CCDC98 with BRCA1 or RAP80. However, we found that both CCDC98-BRCA1 and CCDC98-RAP80 association were DNA damage independent (**Fig. 1**).

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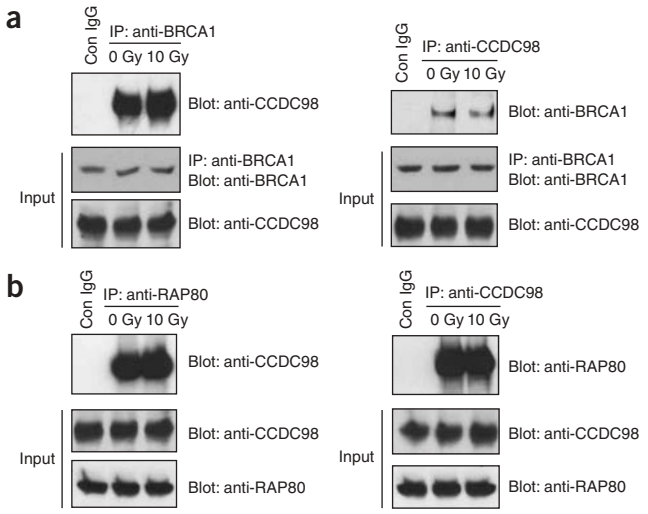

Figure 1 CCDC98 associates with BRCA1 and RAP80. (**a**,**b**) 293T cells were treated with ionizing radiation (10 Gy) or untreated. Cell lysates were subjected to immunoprecipitation (IP) and analyzed by blotting with indicated antibodies to show that CCDC98 associates endogenously with both BRCA1 (**a**) and RAP80 (**b**). Both interactions are DNA damage independent. Whole-cell lysates were analyzed by blotting with indicated antibodies as protein loading controls. Irrelevant IgG was used as immunoprecipitation control (Con).

CCDC98 is the linker between BRCA1 and RAP80

Next, we sought to determine the regions required for binding between the BRCA1-BRCT domain and CCDC98. Recombinant BRCA1-BRCT interacted with endogenous CCDC98 (**Fig. 2a**). BRCA1-BRCT is a phosphoprotein-binding domain[7,8], and when cell lysates were pretreated with λ-phosphatase (λ-PPase) to remove protein phosphorylation, BRCA1-BRCT domain did not recognize CCDC98 (**Fig. 2a**). Moreover, inhibitors that suppress λ-PPase's activity rescued the interaction between the BRCA1-BRCT domain and CCDC98, suggesting that BRCA1-BRCT recognizes phospho-CCDC98.

Previously, we and others have shown that the BRCA1-BRCT domain binds the motif pSXXF (where pS is phosphoserine)[7–9]. The only pSXXF motif in CCDC98's sequence is at the C-terminal end. Using mass spectrometry to screen for phosphorylated sites in BRCA1-BRCT domain–associated CCDC98 (55 CCDC98 peptides covering 85% of its sequence), we confirmed that CCDC98 Ser406 is phosphorylated *in vivo* (data not shown). We raised a polyclonal antibody against a CCDC98 peptide containing pSer406 that recognized only phospho-CCDC98 and not the unphosphorylated form (**Fig. 2a**). Phosphorylated CCDC98 peptide, but not unphosphorylated peptide, blocked recognition of CCDC98 by the antibody to pSer406 (data not shown), supporting the phosphorylated state of CCDC98 *in vivo*. Finally, mutation of Ser406 to alanine almost abolished the interaction with the BRCA1-BRCT domain (**Fig. 2b**). Together, these results suggest that the BRCA1-BRCT domain recognizes pSer406 of CCDC98.

To map the regions where CCDC98 and RAP80 interact, we deleted either the ubiquitin-interacting motif (UIM) or zinc-finger domain of RAP80. Recombinant CCDC98 interacted with wild-type RAP80 but showed almost no interaction with the RAP80 deletion mutants, suggesting that both the UIM and zinc-finger domain are important for RAP80's interaction with CCDC98 (**Fig. 2c**). We also generated a series of internal deletion mutants of CCDC98 on the basis of its structural fold, without disrupting its nuclear localization. Similarly, RAP80 strongly recognized wild-type CCDC98 but weakly associated with CCD98 D1 and D3 mutants and did not interact with the D2 mutant, suggesting that multiple regions in both proteins are required for the RAP80-CCDC98 interaction (**Fig. 2d**).

As CCDC98 interacts with both BRCA1 and RAP80, we examined whether CCDC98 is the mediator between BRCA1 and RAP80. We designed short interfering RNAs (siRNAs) to specifically target *CCDC98*, *RAP80* and *BRCA1*, respectively (**Fig. 3a**). With a control siRNA treatment, RAP80 associated with BRCA1 (**Fig. 3b**). However, downregulation of CCDC98 by *CCDC98* siRNA disrupted the BRCA1–RAP80 complex (**Fig. 3b**), suggesting that CCDC98 is the mediator that bridges the association between BRCA1 and RAP80. In contrast, RAP80 was dispensable for BRCA1-CCDC98 interaction,

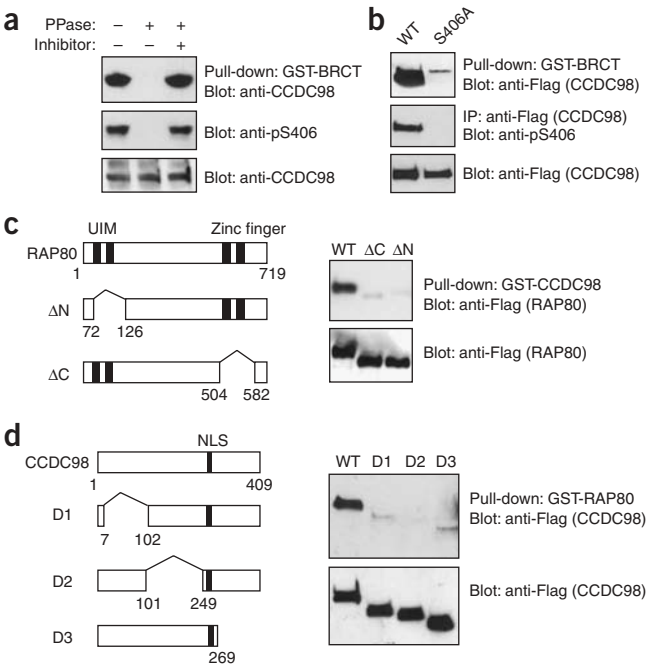

Figure 2 Mapping the interaction domains of BRCA1, CCDC98 and RAP80. (**a**) The BRCT domain recognizes phospho-CCDC98. 293T cell lysates were treated with λ-PPase and/or PPase inhibitors, or untreated. Endogenous CCDC98 was pulled down by GST–BRCA1-BRCT. Whole-cell lysates were also analyzed by blotting with anti-pSer406 to assess the phosphorylation status of CCDC98. A blot with anti-CCDC98 was used as a loading control. (**b**) The BRCA1-BRCT domain recognizes pSer406 of CCDC98. Wild-type CCDC98 or S406A mutant was expressed in 293T cells and pulled down with GST–BRCA1-BRCT. Cell lysates were analyzed by immunoprecipitation and/or blotting with indicated antibodies to examine phosphorylation status of Ser406 and protein expression. (**c**) RAP80 UIM and zinc-finger domain are important for the interaction with CCDC98. Either UIM motif (ΔN) or zinc-finger domain (ΔC) was deleted. Flag-tagged wild-type or mutant RAP80 was expressed in 293T cells. GST-CCDC98 interacts with wild-type RAP80 but not the mutants. Protein expression was examined by blotting with anti-Flag. (**d**) Wild-type CCDC98 and a series of internal deletion mutants, each with a nuclear localization sequence (NLS) and a Flag tag, were expressed in 293T cells. GST-RAP80 pulled down wild-type CCDC98 from cell lysates, but weakly associated with the D1 and D3 mutants and did not interact with D2 mutant. All the constructs were expressed equally, as shown by anti-Flag blotting.

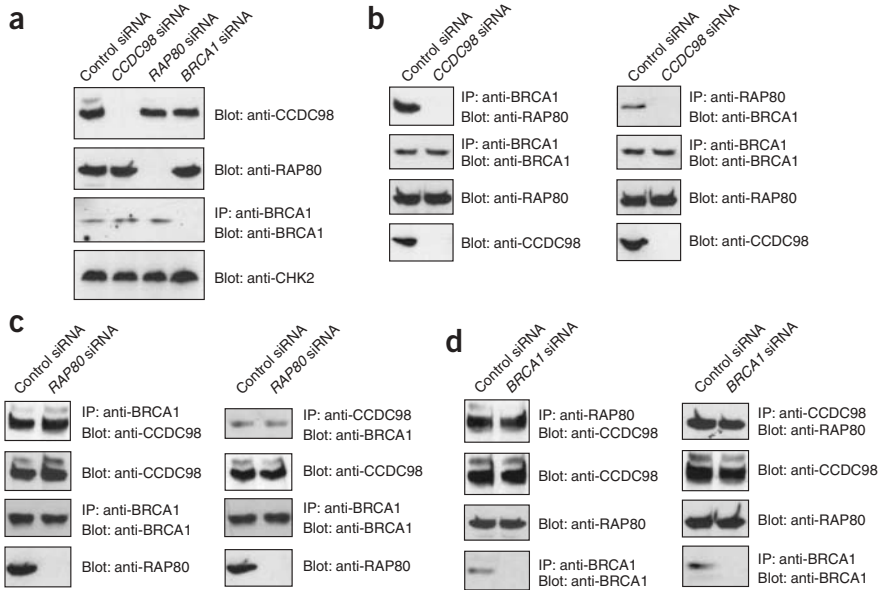

Figure 3 CCDC98 mediates BRCA1-RAP80 association. (**a**) Our siRNAs specifically target *CCDC98*, *RAP80* and *BRCA1*. HeLa cells were treated with control siRNA or siRNA targeting *CCDC98*, *RAP80* or *BRCA1*. Cell lysates were analyzed by blotting with indicated antibodies. The protein loading control was examined with anti-CHK2. (**b**–**d**) siRNA knockdown results show that CCDC98 is the linker between BRCA1 and RAP80 (**b**); that RAP80 does not affect the interaction between BRCA1 and CCDC98 (**c**); and that BRCA1 is dispensable for the interaction between CCDC98 and RAP80 (**d**). HeLa cells were treated with indicated siRNAs to specifically downregulate target proteins, and cell lysates were examined by blotting with indicated antibodies.

and BRCA1 was not required for CCDC98-RAP80 interaction (**Fig. 3c**,**d**). Collectively, our results indicate that CCDC98 is a crucial linker between BRCA1 and RAP80.

CCDC98 targets BRCA1 to DNA damage sites

After cells are exposed to ionizing radiation, BRCA1 translocates to DNA damage sites, colocalizing with γH2AX[31]. We next explored whether the BRCA1-BRCT–binding protein CCDC98 also participates in the DNA damage response. In cells treated with ionizing radiation, CCDC98 formed nuclear foci and colocalized with both γH2AX and BRCA1 (**Fig. 4a**), suggesting that the DNA damage response does involve CCDC98. As the BRCA1-BRCT domain is required for the formation of BRCA1 foci after DNA damage[9,30], we hypothesized that CCDC98 has a key role in targeting BRCA1 to damage sites. Cells without BRCA1 expression retained DNA damage–induced foci of both RAP80 and CCDC98 (**Fig. 4b**), suggesting that both RAP80 and CCDC98 are upstream of BRCA1 in the DNA damage response pathway. When cells were treated with *CCDC98* siRNA to downregulate CCDC98, only BRCA1 foci, and not RAP80 foci, were disrupted (**Fig. 4b**), suggesting that CCDC98 directly loads BRCA1 onto DNA damage sites. Upon treatment with *RAP80* siRNA, both CCDC98 and BRCA1 failed to form DNA damage–induced foci (**Fig. 4b**), further suggesting that CCDC98 is the mediator in the complex between RAP80 and BRCA1 during the DNA damage response. Moreover, mutant CCDC98 bearing an S406A substitution, which abolishes the BRCA1-CCDC98 interaction (**Fig. 2b**), did not target BRCA1 to DNA damage sites (**Supplementary Fig. 1** online), suggesting that the direct interaction between BRCA1 and CCDC98 is required for BRCA1 focus formation after DNA damage.

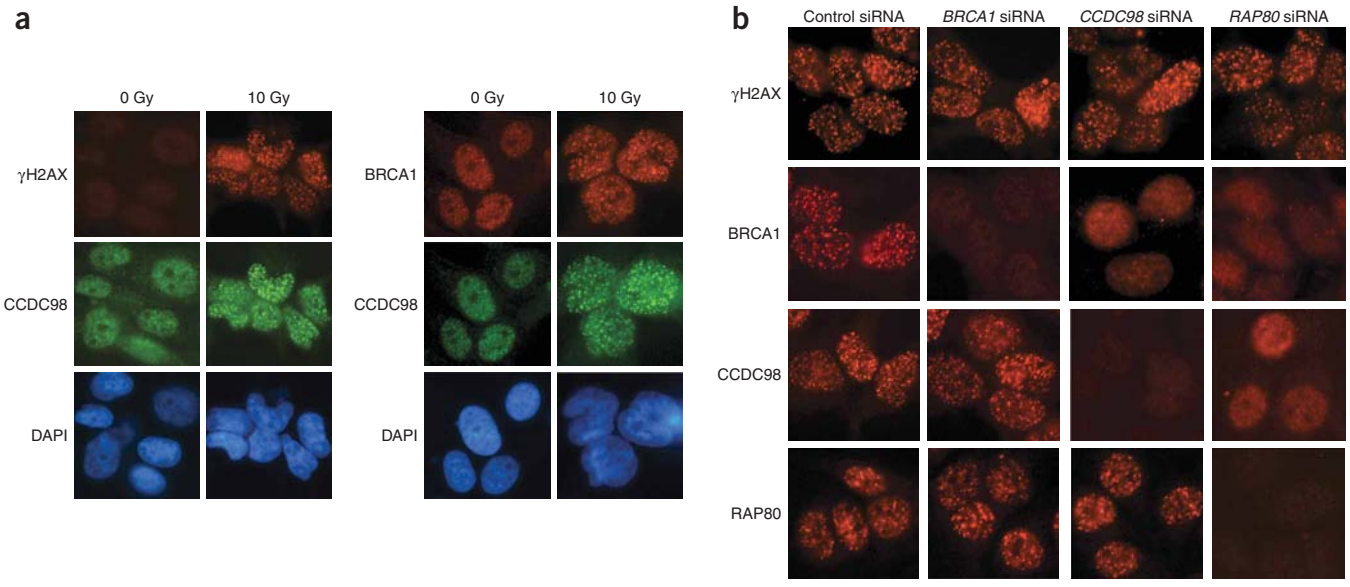

Figure 4 CCDC98 targets BRCA1 to DNA damage sites. (**a**) CCDC98 relocates to DNA damage–induced foci and colocalizes with γH2AX and BRCA1 after induction of DNA double-strand breaks. HeLa cells were treated with ionizing radiation (10 Gy) or untreated, then stained with antibody to γH2AX, BRCA1 and CCDC98. (**b**) CCDC98 is required for BRCA1, but not RAP80, focus formation after DNA damage. HeLa cells were transfected with indicated siRNAs, then treated with 10 Gy ionizing radiation and stained with antibody to γH2AX, BRCA1, CCDC98 or RAP80.

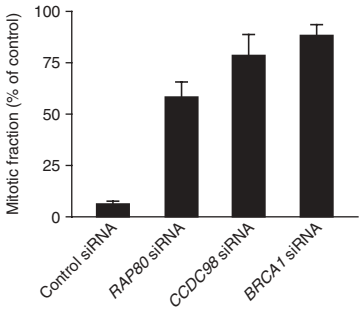

Figure 5 CCDC98 participates in DNA damage–induced G2/M checkpoint activation. HeLa cells were treated with indicated siRNAs, then treated with 2 Gy ionizing radiation or left untreated. The mitotic population was stained with antibody to phosphorylated histone H3 and quantified by FACS analysis. Mitotic fraction in each sample was normalized to that seen in cells without DNA damage (control). Data represent averages from three experiments; error bars show s.d.

CCDC98 participates in G2/M checkpoint activation

As BRCA1–RAP80 complex controls damage-induced G2/M checkpoint activation[40,42], we examined whether CCDC98 also participates in G2/M checkpoint activation. After ionizing radiation treatment, normal cells are arrested in G2 phase, before entry into mitosis, allowing DNA lesions to be repaired. We monitored the mitotic fractions of cultured cells using a mitotic marker, phosphorylated histone H3. When cells were transfected with a control siRNA, their mitotic population was greatly reduced after the induction of DNA double-strand breaks. In contrast, cells treated with either *BRCA1* siRNA or *RAP80* siRNA entered into mitosis regardless of DNA damage (**Fig. 5** and **Supplementary Fig. 2** online), suggesting that both BRCA1 and RAP80 are required to stop cell-cycle progression and activate the G2/M checkpoint after DNA damage. Like *BRCA1* siRNA– or *RAP80* siRNA–treated cells, cells treated with *CCDC98* siRNA also showed no G2/M checkpoint after exposure to ionizing radiation (**Fig. 5** and **Supplementary Fig. 2**), suggesting that CCDC98 has functions similar to those of BRCA1 and RAP80 in DNA damage checkpoint control. Together, these results further strengthen the evidence that BRCA1, CCDC98 and RAP80 form a functional complex and participate in the DNA damage response.

DISCUSSION

BRCA1 is important in the response to DNA damage. In this study, we have identified a new BRCA1-BRCT domain–binding protein, CCDC98, that serves as a functional partner of BRCA1 upon the induction of double-strand breaks in DNA. Another group has also recently identified CCDC98 as a BRCA1-associated protein, calling it Abraxas[42]. Here, we have not only demonstrated that CCDC98 is a crucial mediator that links the BRCA1-BRCT domain with RAP80, but also analyzed the BRCA1–CCDC98–RAP80 complex in detail. The BRCA1-BRCT domain interacts with pSer406 of CCDC98 (ref. 42 and this work), while RAP80 forms a heterodimer with CCDC98 via multiple contact sites. Further structural analysis will be required to understand the organization of the whole complex.

Although the BRCA1-BRCT domain recognizes phospho-CCDC98, the phosphorylation status of Ser406 in CCDC98 is independent of DNA damage (data not shown). Consistent with this observation, the BRCA1-CCDC98 interaction is also DNA damage independent (**Fig. 1**). Previously, two phosphorylation-dependent BRCA1-BRCT domain partners, BACH1 and CtIP, have been characterized. Both the BRCA1–BACH1 and BRCA1–CtIP complexes are involved in the DNA damage response[33,35]. In contrast with these two BRCA1-binding partners, CCDC98 functions upstream of BRCA1 after DNA damage and directly targets BRCA1 to DNA damage sites through its interaction with RAP80. Our results are consistent with previous findings indicating that the BRCA1-BRCT domain is required for BRCA1 focus formation[9,30]. Notably, the UIM of RAP80 is important for formation of foci containing the BRCA1 complex, suggesting that a ubiquitinated protein may recruit the BRCA1 complex to DNA double-strand breaks[40–42]. Nevertheless, targeting of BRCA1 to foci at DNA damage sites could be CCDC98's role in the DNA damage–induced G2/M checkpoint.

In summary, we have identified a complex between CCDC98 and the BRCA1-BRCT domain that is crucial for loading BRCA1 onto DNA damage sites and participates in the DNA damage response. As most breast cancer–associated mutations in *BRCA1* abolish the C-terminal BRCT domain, this complex could be a target for future tumor-prevention studies.

METHODS

Cell culture, antibodies, complementary DNA and short interfering RNA. Cells were maintained in RPMI 1640 media with 10% (v/v) FBS. For ionizing radiation treatment, cells were irradiated using a JL Shepherd ^{137}Cs radiation source at indicated doses. Cells were then returned to the same culture conditions for further analysis. Mouse monoclonal antibody to BRCA1 (SD118) was purchased from Oncogene. Mouse γH2AX-specific monoclonal antibody (JBW301) and rabbit antibody to phospho–histone H3 were from Upstate. Anti-Flag (M2) was from Sigma. Rabbit anti-RAP80 was raised against glutathione *S*-transferase (GST)-fused RAP80 (residues 1–354). Rabbit antibodies to CCDC98 and pSer406 were raised against a CCDC98 peptide (CKGFEYSR-pS-PTF). Full-length *CCDC98* cDNA from IMAGE clone 6045433 was subcloned into pS-Flag-SBP vector encoding an IRES-EGFP fluorescent tag. Full-length RAP80 was also cloned into pS-Flag-SBP vector, as described[40]. BRCA1-BRCT domain, CCDC98 and RAP80 were all cloned into pGEX 4T-1 vector (Amersham) to generate GST fusion proteins. The siRNA sequences targeting *BRCA1*, *CCDC98* and *RAP80* are 5′-GGAACCU GUCUCCACCAAAGdTdT-3′, 5′-GUAAAAGGUGAAGCCAAGAdTdT-3′ and 5′-GAAGGAUGUGGAAACUACCdTdT-3′, respectively.

Protein affinity purification. BRCA1-BRCT domain (residues 1599–1863) and full-length RAP80 were cloned into pS-Flag-SBP vector. K562 cells stably expressing BRCA1-BRCT or RAP80 were selected. Cells were harvested from 11 of culture and lysed with 30 ml NETN buffer (0.5% (v/v) Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 100 mM NaCl). The soluble fraction was incubated with 500 μl streptavidin-conjugated beads, and associated proteins were eluted with 2 mM biotin and further incubated with 50 μl S beads (Novagen). The bound proteins were analyzed with SDS-PAGE and mass spectrometry. To search for phosphorylation sites of CCDC98, we analyzed a 50-kDa band from the BRCA1-BRCT domain purification by mass spectrometry. For further details, see **Supplementary Methods** online.

Immunoprecipitation, GST pull-down assay and western blotting. Cells were lysed with NETN buffer. The soluble fraction was treated with λ-PPase and/or PPase inhibitors as described[7], or left untreated. Immunoprecipitation, GST pull-down assays and western blotting were done as described[7].

Immunofluorescence staining. Cells were treated with or without 10 Gy ionizing radiation. Two hours after treatment, cells were fixed with 3% (w/v) paraformaldehyde, permeabilized with 0.5% (v/v) Triton-100 and stained with the indicated antibodies. To study the dominant-negative effects of the S406A mutant, S-Flag-SBP–tagged wild-type CCDC98 or S406A mutant was transiently expressed in HeLa cells. We monitored EGFP expression to identify positive transfectants.

G2/M checkpoint assay. Cells were transfected with the indicated siRNAs twice. Forty-eight hours after the second transfection, cells were treated with

2 Gy ionizing radiation. One hour later, cells were fixed with 70% (v/v) ethanol and stained with anti–phospho–histone H3 (Ser10) and propidium iodide. Cells were analyzed by FACScan. The assay was repeated three times. We quantified FACS results by comparing the mitotic fraction of cells with and without DNA damage treatment, setting the mitotic population without ionizing radiation to 100%.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

X.Y. purified proteins and identified the BRCA1–CCDC98–RAP80 complex. Z.L. and J.W. analyzed the protein interactions of this complex. Z.L. and X.Y. examined the role of CCDC98 in the formation of DNA damage–induced protein foci and G2/M checkpoint activation. X.Y. drafted the manuscript. All the authors read and approved the final manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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